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Hypoxia, Mn-SOD and H_2O_2 regulate p53 reactivation and PRIMA-1 toxicity irrespective of p53 status in human breast cancer cells

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ABSTRACT

Hypoxia is part of the tumor microenvironment favoring cancer resistance to chemotherapy mediated by mutations in the tumor suppressor p53 gene (TP53), or by conformational wt TP53 dysfunction. Since it is important to suppress tumor adaptation to hypoxia, irrespective of p53 status, we compared the efficacy of nutlin-3 which prevents MDM2-wt p53 interactions and PRIMA-1 which promotes mutant p53 reactivation and induction of massive apoptosis, under normoxia and hypoxia, against (a) SKBR3 breast carcinoma harboring a mutant p53R175H and over-expressing erbB2; and (b) genetically matched breast cancer ERα positive MCF-7 cells harboring either wt p53 or mutant p53 R175H. Under normoxia, PRIMA-1 was active against breast cancer cells harboring mutant p53. However, hypoxia further increased the susceptibility of mutant p53 breast cancer SKBR3 cells to lower PRIMA-1 levels, possibly through oxidative stress since this was counteracted by N-acetylcysteine. When using MCF-7 cells over-expressing mutant p53, PRIMA-1 synergized with exogenous peroxidase to increase apoptosis concomitantly with induction of PUMA and Mn-SOD, under normoxia. Wt p53 MCF-7 cells responded to hypoxia by increasing superoxide dismutase and their reactivity with the PAb240 antibody, known to recognize conformationally-inactive p53. This correlated with sensitization of wt p53 MCF-7 cells to PRIMA-1 but not to nutlin-3. PRIMA-1 toxicity against normoxic wt p53 MCF-7 cells was also decreased by Mn-SOD over-expression or when added with the glutathione antagonist, buthionine sulfoximine. This report shows for the first time that hypoxia increases PRIMA-1 toxicity in human breast cancer cells, partly by modulating p53 conformation and by inducing superoxide turnover. Our results suggest that PRIMA-1 may help to prevent hypoxia-mediated tumor chemoresistance.

1. Introduction

During cancer progression, tumor cells face a progressively hypoxic and hypoglycemic microenvironment when growing distant from sites of vascularization [1,2]. Hypoxia originates a gradient of oxidative stress mediated by H_2O_2 capable of promoting cancer cell heterogeneity, by enhancing mutational potential [2], which helps select cells with resistance to conventional anti-tumor therapies [3–7]. Genomic instability of cancer cells is frequently antagonized by activation of the tumor suppressor protein encoded by the Tp53 gene, which is involved in monitoring DNA damage to help induce growth arrest and DNA repair or apoptosis, to help maintain the integrity of genome [8,9]. Many cancer cells die in response to hypoxia because their high metabolic requirements make them much more dependent on glycolytic pathways which increase their glucose demands [10,11]. However, tumor cell adaptation to hypoxic stress partly occurs because p53 tumor suppressive function may be compromised by the stress induced by sub-optimal glucose and oxygen [12–14] or by oxidative stress [15]. Hence, it is important to find conditions to enhance activation of wt p53 and to re-activate mutant p53 in heterogeneous tumor cell populations, in which low oxygen and oxidative imbalance could inactivate Tp53 either by mutation [8,9] or conformational dysfunction [13–16]. The small molecule PRIMA-1 [bis(hydroxymethyl)-3-quinuclidinone], was discovered by its ability to promote mutant p53 reactivation and induction of massive apoptosis in various cancer cells [17]. Although the effect of PRIMA-1 and its analog PRIMA-1^{Met} is frequently recognized as specific for mutant p53 cells [17–22], more recently, PRIMA-1^{Met} was found effective against small cell lung carcinoma cells with low or undetectable levels of mutant p53 [19]. In some breast cancer cells, PRIMA-1 induced cell death in vitro and in vivo in estrogen-responsive cell lines that express mt p53 (BT-474,

Abbreviations: DN-mutant p53 R175H, dominant-negative mutant p53 Arg 175His; wt, wild type; PARP, poly(ADP-ribose) polymerase; [Mn-SOD, SOD2], manganese-dependent superoxide dismutase; [Cu/Zn-SOD, SOD1], copper–zinc-dependent superoxide dismutase; BSO, buthionine sulfoximine; ROS, reactive oxygen species; NAC, N-acetylcysteine; GSH, glutathione.

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HCC-1428, and T47-D) [20]. In contrast, PRIMA-1 had no effect on the viability of MCF-7 cells, normal breast cells, and endothelial cells, all of which express wild-type 53 protein [20]. PRIMA-1 also induced apoptosis in human breast cancer cells MDA-MB-231 and GI-101A, mediated by PUMA and Bax induction [21]. Although several reports implied the selectivity of PRIMA-1 for mutant p53 cells, it was recently shown that PRIMA-1^{Met} binds not only to mutant p53, but it can also bind to unfolded wt p53 [22], with the degree of binding correlating with the extent of unfolding, implying that PRIMA-1^{Met} may also activate unfolded wt p53 [22]. Since hypoglycemic/hypoxic condition mimicking in vitro the tumor microenvironment markedly reduced the efficacy of anticancer drugs [1], we have now compared the hypoxic and normoxic response of some human breast cancer cells to PRIMA-1 and to an alternative p53-reactivating drug, nutlin-3, known to act by preventing wt p53 degradation by disrupting the p53–MDM2 interactions [23–26]. However, it is of interest that the ability of nutlin-3 to activate p53 is compromised in tumor cells over-expressing MDMX, like MCF-7 cells [24,25]. However, since nutlin-3 radiosensitizes hypoxic prostate cancer cells independent of p53, it seemed of interest to investigate how nutlin-3 and PRIMA-1 reacted against MCF-7 cells under hypoxia since this [10,12–14] and oxidative stress [15] can induce an inactive conformation in wt p53 [16]. Initially, we used SKBR3 breast carcinoma harboring endogenous mutant p53R175H and erbB2//Her2 over-expression [27,28]. These cells are important because of the human epidermal growth factor receptor 2 (HER2) and p53 pathways may be involved in breast cancer response to chemotherapy [29]. We also investigated the responses to PRIMA-1 in genetically-matched ERα^+ human breast cancer MCF-7 cells expressing wt p53 or an exogenous mutant p53R175H [30], and in wt p53 MCF-7 cells over-expressing Mn-SOD and the corresponding vector-transfected cells [31]. We also investigated whether hypoxia partly induced a conformational unfolded p53 protein recognized by the monoclonal antibody PAb240 (directed toward unfolded p53) [15,16]. Moreover, since it is important to improve the efficacy of PRIMA-1 against mutant p53 SKBR3 breast carcinoma, we also studied whether hypoxia also enhances their response to lower PRIMA-1 concentrations. Finally, since restriction of oxygen at may increase superoxide metabolism [32], we also investigated whether these conditions influence superoxide dismutase (SOD) expression.

2. Materials and methods

2.1. Cell lines

Experiments were performed with: (a) SKBR3 harboring endogenous mutant p53R175H and erbB2//Her2 over-expression [27–29]; (b) wt p53 MCF-7 human breast carcinoma neo^4 cells, obtained by transfection with control Neo-vector, and Mn-SOD-over-expressing (Mn11-cells) transfected with Mn-SOD cDNA in the same vector. These cells were cultured in Dulbecco's medium containing 10% fetal bovine serum, and G418 at 400 μg/ml. The verification of Mn-SOD over-expression for Mn11 cells was previously described [31]. We appreciate the kind provision of Mn11- and Neo-MCF-7 cells by Dr. L. Oberley, Free Radical and Radiation Biology Program, Department of Radiation Oncology, University of Iowa, Iowa City, Iowa, USA; (c) MCF-7 human breast carcinoma cells which harbor a functional wt p53 or a mutant p53 R175H [30]. To obtain isogenic wt p53 or mutant p53 cells, parental MCF-7 cells were transduced with plasmids (kindly provided by Dr. Scott Lowe, Cold Spring Harbor Laboratory, New York) packaged into Phoenix retroviral packaging cells (kindly provided by Dr. Garry P. Nolan, Stanford University, CA). Phoenix cells were transfected with the pWZL-Hygro plasmid harboring a human p53 histidine-175 mutant gene, a dominant-negative (DN) p53 mutant plasmid, in the presence of calcium chloride. As previously described [30], control cells were retrovirally transduced with the empty pWZL-Hygro plasmid. Hygromycin (Calbiochem, San Diego, CA) was used at a final concentration of 100 μg/ml. Verification of conformationally inactive p53 was achieved by immune precipitation of mutant p53 with monoclonal antibody Pab 240 (SC-99), p53 mutant-specific under nondenaturing conditions or by staining cells fixed with 4% p-formaldehyde in PBS, followed by cell permeabilization with non-ionic detergents like octyl glucoside or Nonidet P40 at 0.5%. Conformationally active wt p53 was identified by lack of immune precipitation with Pab 240 (SC-99) under nondenaturing immune precipitation and reactivity with monoclonal antibody (DO-1; SC-126) under comparable conditions. Immune precipitations were aided by the addition of protein A/G Plus agarose (SC-2003). Precipitated proteins were subjected to SDS-PAGE electrophoresis, bidirectionally blotted onto nitrocellulose membranes and identified by DO-1 (SC-126) monoclonal antibody, which recognizes both wt and mutant denatured p53's. All antibodies and protein A/G Plus agarose were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Relative cellular metabolic activity

This was estimated with Alamar Blue (resazurin) obtained from Life Technologies (Carlsbad, CA). It measures intracellular redox mitochondrial activity by quantitating the cell-catalyzed conversion of non-fluorescent resazurin to fluorescent resorufin. For these experiments, cells (10,000) were allowed to adhere overnight in 96 well TC microtiter dishes. After the corresponding treatments, Alamar Blue was added to 10% of the cell volume without removing medium containing dead cells, and fluorescence was measured 4 h later in a Labsystems Fluoroskan Ascent microplate reader at an excitation of 544 nm and an emission of 590 nm [33].

2.3. Western blot analysis

Cells were harvested in PBS containing protease and phosphatase inhibitors using a rubber policeman. Extracts were prepared in cell lysis buffer (50 mM Tris–HCl, pH 8, 120 mM NaCl, 50 mM NaF, 0.1 mM sodium vanadate, 5 mM EDTA, 10 μg/ml each of leupeptin, soybean trypsin inhibitor, and aprotinin, 1 mM phenylmethylsulfonyl fluoride, 0.4% Nonidet P40). Seventy-five micrograms of protein was loaded into each well of a 11% SDS-polyacrylamide gel and electrophoretically separated. After protein transfer, the membranes were blocked with TBS (Tris-buffered saline, pH 7.5) containing 0.1% Tween-20 and 5% nonfat skim milk. All the chemicals above indicated were obtained from Sigma–Aldrich (St. Louis, MO, USA). Antibody detecting both the intact and cleaved PARP forms [33] and antibody versus actin were from Cell Signaling (Waltham, MA, USA).

2.4. Crystal violet staining of surviving adherent cells

Cells were subjected to the treatments indicated in each case under hypoxia or normoxia. Subsequently, the unattached dead population was removed after washing twice in isotonic phosphate-buffered saline. Surviving cells were evidenced following fixation in 90% ethanol and cell staining with 0.5% crystal violet in 30% ethanol (both from Sigma–Aldrich, St. Louis, MO, USA). Whenever indicated, quantitation was achieved at 595 nm after eluting the dye in 30% ethanol from quadruplicate stained cells [33].

2.5. Laser scanning cytometry

A laser scanning cytometer (LSC)-2 (Compucyte, Cambridge, MA), which measures fluorescence intensity of individual cells

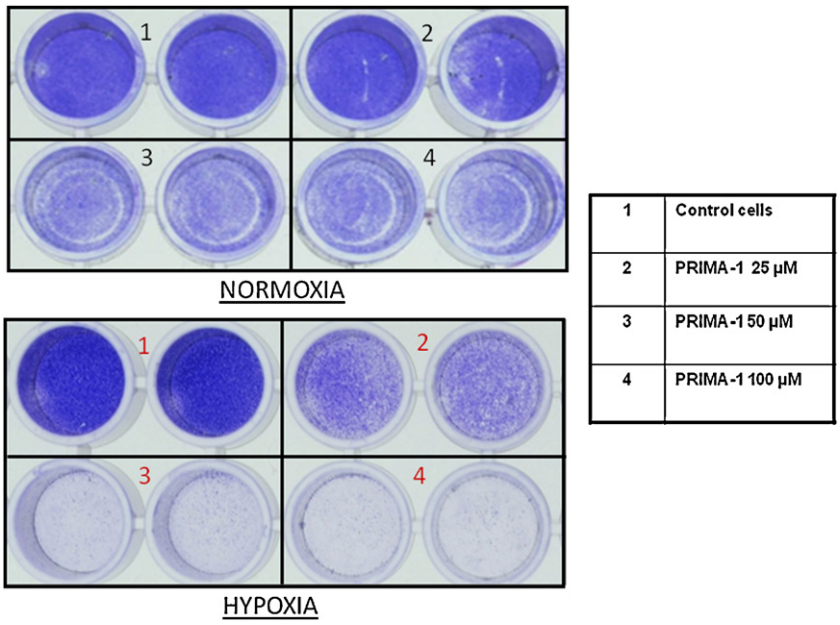

Fig. 1. Hypoxia increases PRIMA-1 toxicity against SKBR3 breast cancer cells. Cultures were seeded in quadruplicate at 1×10^4 cells per 96 well plate overnight in complete Dulbecco's medium with 20 mM glucose supplemented with 10% fetal calf serum medium. The following day, cells were exposed to normoxia or hypoxia (≤1% oxygen) including 0.1% DMSO (controls) or PRIMA-1 at the indicated concentrations in 0.1% DMSO. After 72 h, cells were fixed in 70% ethanol followed by crystal violet staining, as indicated under Section 2. Note the progressive loss of cell viability evidenced by decreased staining particularly evident in PRIMA-1 treated cells under hypoxia.

contoured on the basis of nuclear DNA counterstain with propidium iodide was used. Every sample was scanned using identical nonsaturating fluorescence settings, to allow quantitative comparisons to be made. To analyze fluorescence changes on individual cells, clustered aggregates were gated, so as to include as many individual contoured cells as possible and quantitate integral (total fluorescence within the integral contour) and maximal pixels (highest localized fluorescence within the threshold contour). After the indicated culture conditions, cells attached to LabTek multiwell plates (Nunc-Nalge, Naperville, IL) pre-coated with fibronectin were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), followed by permeabilization with 0.25% Nonidet P40, washing in PBS, blocking in 5% albumin (in PBS). All these reagents were obtained from Sigma–Aldrich (St. Louis, MO). After fixation and permeabilization, cells were reacted with either: (i) monoclonal antibody recognizing p53-serine 15-phosphorylation from Cell Signaling (Waltham, MA, USA) or (ii) monoclonal antibody to conformationally inactive Pab 240 (SC-99) (Santa Cruz Biotechnology, Santa Cruz, CA). Detection of green fluorescence was achieved by reaction with corresponding secondary antibodies conjugated to Oregon Green (Molecular Probes, Eugene, OR) for excitation with an Argon laser. Integral contouring was done by staining DNA with propidium iodide (Sigma–Aldrich, St. Louis, MO) in the long red region.

3. Results

3.1. Hypoxia increases PRIMA-1 toxicity against mutant p53 SKBR3 breast cancer cells

The human epidermal growth factor receptor 2 (erbB2/HER2) and p53 pathways may be involved in the therapeutic response of breast cancer cells. Since PRIMA-1 is known to primarily reactivate mutant p53, we initially investigated whether the toxicity of this agent differed under normoxia or hypoxia against SKBR3 breast cancer cells that endogenously harbor a mutant p53R175H gene and over-express HER2 [27,28]. After seeding these cells overnight in complete Dulbecco's medium containing 20 mM glucose and 10% fetal bovine serum, PRIMA-1 was added at three different concentrations in 0.1% DMSO, adding the same vehicle to the corresponding controls, for a 3 day exposure to normoxic or hypoxic conditions. Crystal violet staining of surviving cells revealed that normoxic cells were partly susceptible to 50 μM PRIMA-1, but essentially resistant to 25 μM PRIMA-1. However, PRIMA-1 cytotoxicity was clearly enhanced under hypoxia at all concentrations tested, including 25 μM (Fig. 1).

3.2. Glucose-dependent SKBR3 cell survival under hypoxia in 2% serum is counteracted by 25 μM PRIMA-1

Since tumor cells face glucose and growth factor shortage when growing distant from sites of vascularization [1], we investigated the response to PRIMA-1 and to nutlin-3, another p53-reactivating small molecule [23–26] in SKBR3 cells exposed to low serum and lower glucose concentrations [1] instead of those used for sub-culturing proliferating tumor cells such as 20 mM glucose and 10% fetal bovine serum. For this, we used 2% dialyzed serum with glucose supplementation between 1.25 mM and 10 mM glucose, adding PRIMA-1 and/or nutlin-3, at the concentrations indicated. SKBR3 cells did not grow significantly under physiological 5 mM glucose in normoxia but showed viability at 10 mM glucose under hypoxia. This was specifically counteracted by 25 μM PRIMA-1, when used singly or together with nutlin-3, with no comparable single response to nutlin-3 (Fig. 2A). In contrast, under normoxia, SKBR3 cells in 2% dialyzed serum showed a glucose-dependent survival, unaffected by PRIMA-1 even when supplemented with nutlin-3 (Fig. 2B).

3.3. Synergism between PRIMA-1 and hypoxia leads to apoptosis-associated PARP cleavage mediated by activation of p53-serine 15 phosphorylation

To investigate some of the mechanisms involved in the potentiation of PRIMA-1 activation by hypoxia, we used SKBR3 cells cultured for 3 days in medium supplemented with 5% dialyzed fetal calf serum and physiological 5 mM glucose under

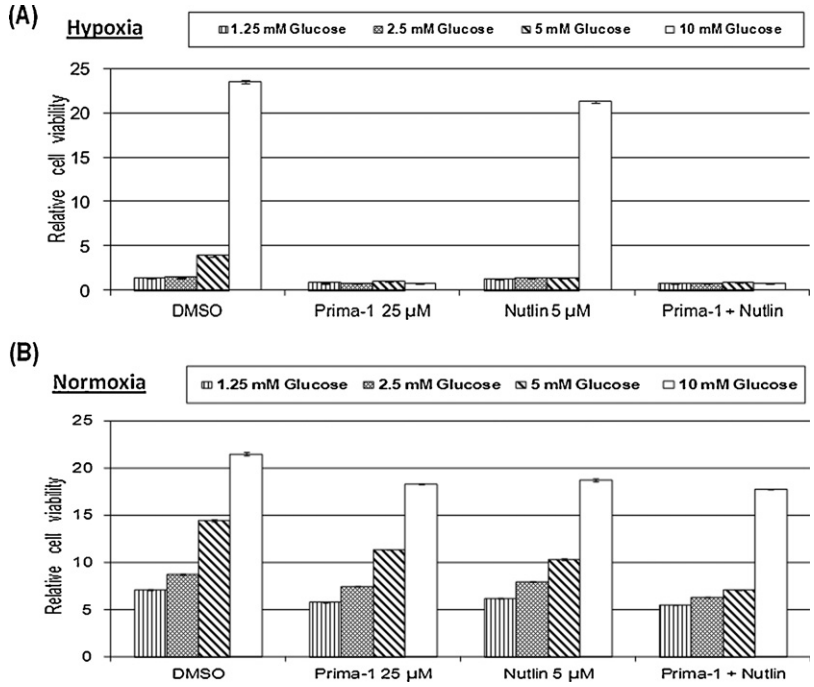

Fig. 2. SKBR3 cell survival after 4 days in hypoxia and 2% serum is promoted by glucose and counteracted by 25 μM PRIMA-1. Cells were seeded overnight as quadruplicates in Dulbecco's medium supplemented with 20 mM glucose and 10% fetal calf serum. After extensive washing, the medium was replaced with glucose-free medium supplemented with 2% dialyzed serum, including glucose, DMSO, PRIMA-1 in 0.1% DMSO and/or nutlin-3 in 0.1% DMSO, and cells were treated for 96 h, as indicated in each experiment. Note that cell survival was glucose-dependent and suppressed whenever 25 μM PRIMA-1 was present under hypoxia, unless N-acetylcysteine (NAC) was present (A). No comparable PRIMA-1 toxicity was detected when cells were exposed to the same treatments under normoxia (B).

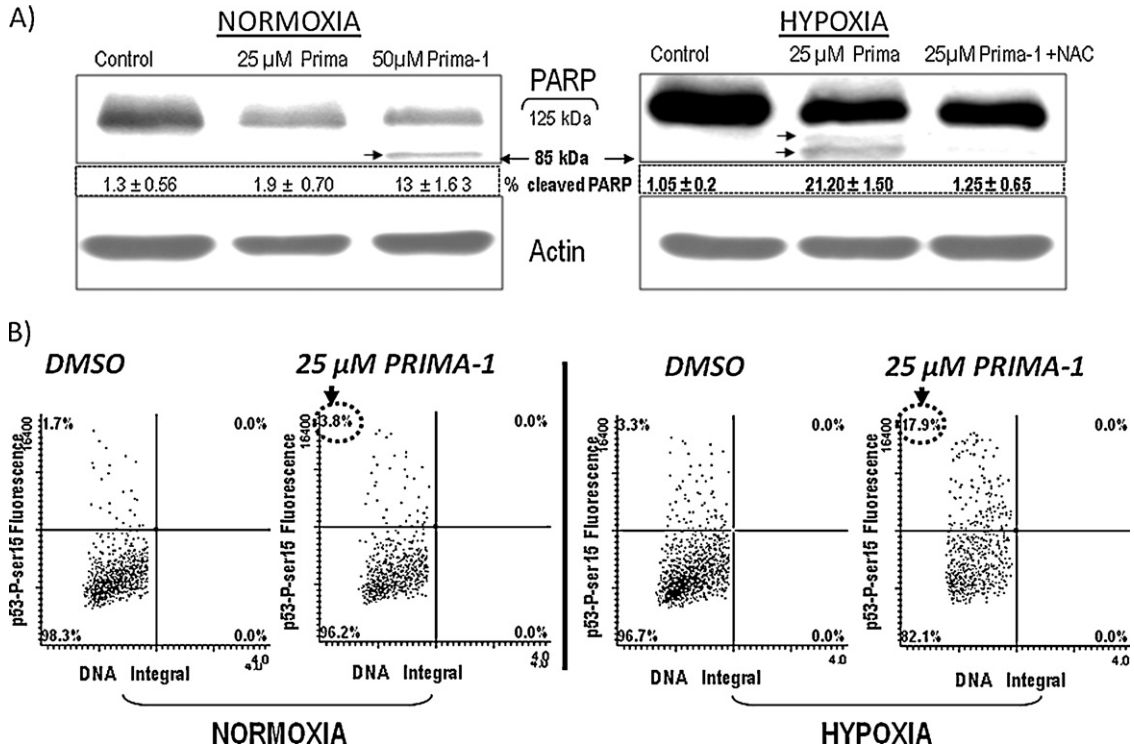

Fig. 3. (A) Apoptosis-associated PARP cleavage induced by 25 μM PRIMA-1 in hypoxic SKBR3 cells is counterated by NAC. SKBR3 cells cultured overnight in complete medium were extensively washed to remove serum and glucose for further culture for 72 h, under normoxia or hypoxia, in medium supplemented with 10 mM glucose and 5% dialyzed serum in the presence of 25 μM or 50 μM PRIMA-1, including 2 mM N-acetylcysteine (NAC) whenever indicated. Cells were collected for immune blotting, to assay the relative ratio of intact PARP to apoptosis-associated PARP fragment (indicated by small arrows). Results were normalized to relative actin levels. (B) 25 μM PRIMA-1 preferentially increases p53-serine 15-phosphorylation in hypoxic SKBR3 cells. Cells cultured as indicated above were exposed to 25 μM PRIMA-1 under hypoxia or normoxia in medium supplemented with 10 mM glucose and 2% dialyzed serum whenever indicated, for 72 h. Subsequently, cells were fixed with 4% p-formaldehyde in phosphate-buffered saline pH 7.2 (PBS), permeabilized in 0.5% Nonidet P40 in PBS, reacted with monoclonal antibody to detect p53-serine 15-phosphorylation, followed by staining with the respective Alexa Fluor 488–secondary antibody conjugate and the DNA fluorochrome, propidium iodide, for laser scanning cytometric analysis.

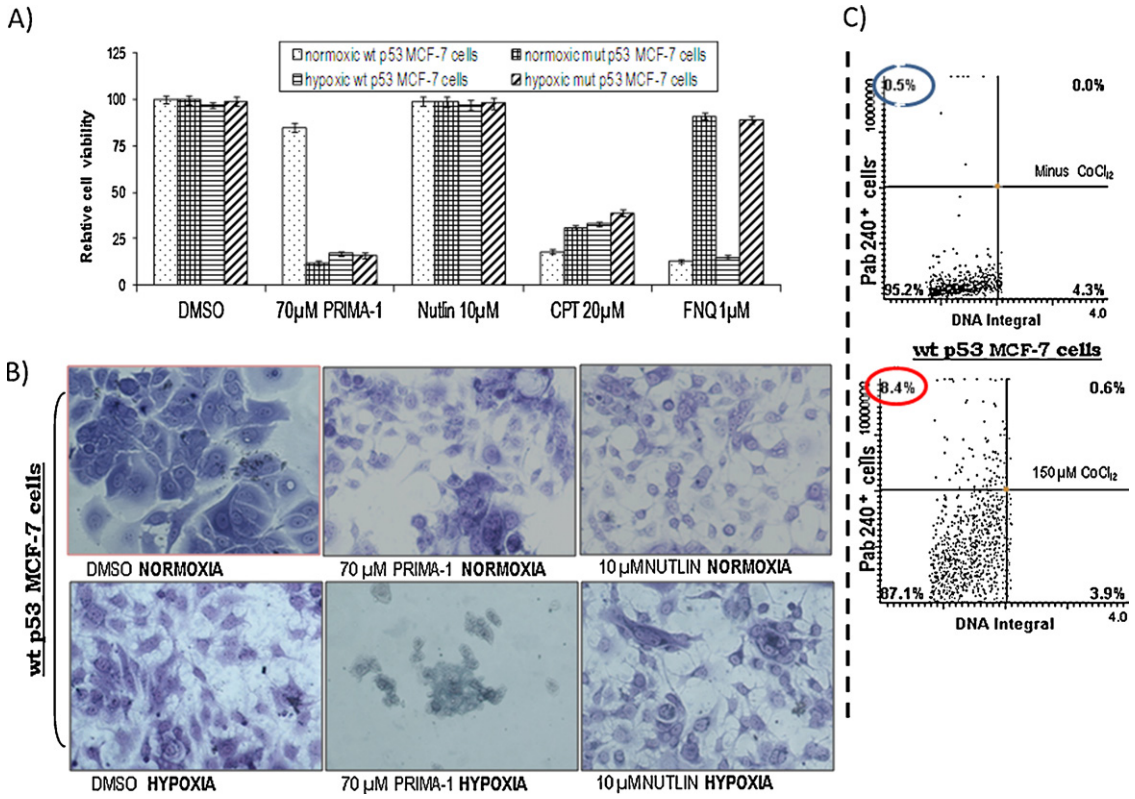

Fig. 4. Hypoxia increases reactivity with PAb240 and counteracts resistance of wt p53 MCF-7 breast cancer cells to PRIMA-1. (A) Genetically matched MCF-7 cells differing in p53 genotypes were seeded in quadruplicate at 1×10^4 cells per 96 well plate overnight in complete Dulbecco's medium with 20 mM glucose supplemented with 10% fetal calf serum. The following day, cells were exposed to normoxia or hypoxia (≤1% oxygen) for 72 h with 0.1% DMSO (controls) or PRIMA-1, nutlin-3, camptotehecin (CPT) or 2-acetyl furanonaphtoquinone (FNQ) at the indicated concentrations in 0.1% DMSO. Live-dead analysis and DNA levels were quantitated in an isocyte laser scanning. spectrofluorimeter. (B) Cells treated as above indicated were fixed in 70% ethanol followed by crystal violet staining as indicated under Section 2. Note the morphological changes induced, and the loss of cell viability in wt p53 MCF-7 cells after PRIMA-1 treatment under hypoxia. (C) Chemical hypoxia inducer $CoCl_2$ increases reactivity with Pab 240 in wt p53 MCF-7 cells.

normoxia or hypoxia. Fig. 3A revealed that normoxic cells require 50 μM PRIMA-1 to induce PARP cleavage, in contrast to 25 μM PRIMA-1 in the corresponding hypoxic cells. The potentiation of PRIMA-1 toxicity under hypoxia was antagonized by the anti-oxidant N-acetylcysteine (NAC). This correlated with a greater activation of p53-serine 15 phosphorylation [21] in hypoxic cells exposed to 25 μM PRIMA-1 (Fig. 3B).

3.4. Hypoxia induces a conformationally inactive epitope in wt p53 MCF-7 cells and increases their susceptibility to PRIMA-1

Since PRIMA-1^{Met} can bind to mutant p53, but it can also bind to unfolded wt p53 implying that PRIMA-1^{Met} could activate unfolded wt p53 [22,19], we used laser scanning cytometry to determine whether $CoCl_2$, an inducer of chemical hypoxia, increased the mutant p53 conformation in wt p53 MCF-7 ERα cells. This revealed an increased binding of the Pab 240 monoclonal antibody directed to a conformationally inactive p53 epitope exposed preferentially by mutant p53 (Fig. 4C). Based on our above observations, we also investigated whether hypoxia increased the toxicity of PRIMA-1 against MCF-7 ERα breast cancer cells, irrespective of their p53 status. For this, genetically-matched MCF-7 cells harboring wt p53 or mutant p53, were seeded overnight in complete medium with 10% fetal bovine serum, followed by a 2 day exposure to PRIMA-1, nutlin-3, camptothecin (CPT) or 2-acetylfuranonaphthoquinone (FNQ) [34] under normoxia or hypoxia. This revealed uniform CPT toxicity in both cell types and resistance of mutant p53 MCF-7 cells to FNQ. Consistent with other reports [24,25], the MDM2 inhibitor nutlin-3 failed to promote apoptosis in normoxic MCF-7 cells, although it is known to cause a significant reduction in the percentage of S-phase cells in MCF-7 cultures, at the same concentration used in other studies [24,25]. However, it was noteworthy that hypoxia counteracted the resistance of wt p53 MCF-7 cells to PRIMA-1, in contrast to the susceptibility of matched mutant p53 MCF-7 cell counterparts to the same treatment (Fig. 4A and B). In contrast, hypoxia did not enhance nutlin-3 toxicity against genetically-matched MCF-7 cells irrespective of p53 status (Fig. 4A and B). In spite of the apparent resistance of wt p53 MCF-7 cells to PRIMA-1 or nutlin-3 in normoxia, these cells changed their normoxic epithelial morphology when exposed to these agents, without evidence of overt toxicity. Moreover, even without any additions, hypoxia disrupted the typical epithelial cobblestone appearance of MCF-7 cells [30], inducing a more mesenchymal organization (Fig. 4B). On the contrary, hypoxic wt p53 MCF-7 cells treated with PRIMA-1 showed loss of viability (Fig. 4A and B).

3.5. PRIMA-1 increases SOD2 expression in hypoxic wt p53 MCF-7 cells

PRIMA-1 and PRIMA-1^{Met} possibly promote an oxidative environment in tumor cells, in which they may be converted to compounds that form adducts with thiols in mutant p53 [22]. Since Mn-SOD (SOD2) can protect against superoxide generated in mitochondria, [31], we investigated whether Mn-SOD (SOD2) is induced by hypoxia and PRIMA-1. The relative expression of Mn-SOD (SOD2) was quantitated in wt p53 MCF-7 cells seeded in 96 well plates. After hypoxia or normoxia, including PRIMA-1 whenever indicated, fixed and permeabilized cells were reacted with a monospecific antibody which identified a single SOD2 band

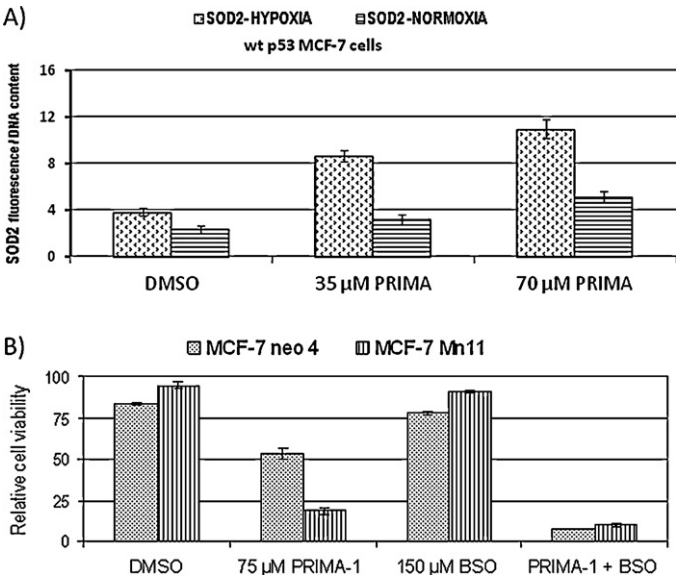

Fig. 5. (A) PRIMA-1 increases SOD2 expression in hypoxic wt p53 MCF-7 cells. wt p53 MCF-7 cultures were exposed to hypoxia in quadruplicates in 96 well plates, including PRIMA-1 whenever indicated for 24 h. Cells were fixed, permeabilized and stained with antibody against SOD2, as indicated under Section 2. Detection was achieved following addition of the respective Alexa Fluor 488–secondary antibody conjugate for green staining, and propidium iodide, for DNA red staining. The ratio of specific green fluorescence normalized to red DNA fluorescence was quantitated in an isocyte laser scanning spectrofluorimeter. (B) SOD2 over-expression and BSO increase PRIMA-1 toxicity against wt p53 MCF-7 cells. neo^4 cells and Mn-SOD-over-expressing Mn11 cultures, originated from wt p53 MCF-7 cells [31] were seeded in sextuplicate at 1×10^4 cells per 96 well plate overnight in complete Dulbecco's medium with 20 mM glucose supplemented with 10% fetal calf serum medium. The following day, cells under normoxia were exposed to DMSO (controls), PRIMA-1 and BSO whenever indicated. Relative cell viability was determined after 72 h by quantitating the cell-catalyzed conversion of non-fluorescent resazurin to fluorescent resorufin, as indicated under Section 2.

by specific immune blotting (not shown). Following removal of unbound antibody by extensive washing, the respective Alexa Fluor 488-conjugated secondary antibody was added to provide green staining and propidium iodide was included to detect DNA red staining. Quantitation of relative green SOD fluorescence versus red DNA content indicated that hypoxia and PRIMA-1, increases SOD2 expression in wt p53 MCF-7 cells (Fig. 5A).

3.6. SOD2 over-expression and BSO increase PRIMA-1 toxicity against wt p53 MCF-7 cells

To gain additional insight into a role for Mn-SOD in PRIMA-1 toxicity, MCF-7 neo^4 cells and Mn-SOD-over-expressing Mn11 MCF-7 cells [31] were used to determine whether PRIMA-1 toxicity under normoxia was influenced by Mn-SOD expression. This revealed greater susceptibility to 75 μM PRIMA-1 in Mn-SOD overexpressing Mn11 cells compared to control neo^4 cells (Fig. 5B). The role of glutathione in the response to PRIMA-1 was also investigated by examining the response to buthionine sulfoximine (BSO), a glutathione-depleting agent [35,36]. This showed that BSO by itself did not affect cell viability. However, it potentiated PRIMA-1 toxicity in both normoxic control neo^4 and Mn11 cells (Fig. 5B).

3.7. PRIMA-1 synergizes with peroxidase to promote mut p53-MCF-7 apoptosis

Since NAC counteracted the hypoxia-induced apoptosis in SKBR3 at 25 μM PRIMA-1 (Fig. 3A), and BSO increased PRIMA-1 toxicity against MCF-7 cells (Fig. 5B), this implicared redox imbalance in PRIMA-1 toxicity, in agreement with others showing that PRIMA-1 and PRIMA-1^{Met} react covalently with thiol groups in mutant p53 [22]. Hence, we investigated in mutant p53-MCF-7 cells, whether the toxicity of PRIMA-1 was modulated by exogenous addition of either peroxidase (PRX) or superoxide dismutase (SOD) at 250 μg/ml. Cells were cultured for 20 h in complete Dulbecco's medium supplemented with 10% fetal bovine serum. Subsequently, PRX or SOD was added overnight followed by PRIMA-1 treatment whenever indicated for 1 additional day. Electrophoretic separation and specific immune blotting revealed that apoptosis-associated PARP cleavage was enhanced in cells jointly treated with PRX + PRIMA-1, although PRX also caused some PARP cleavage. In contrast, no significant PARP fragmentation was seen in cells jointly treated with SOD and PRIMA-1. Maximal PARP degradation induced by PRX and PRIMA-1 correlated with a relative increase in Puma, suggestive of mutant p53 reactivation [21] and super-induction of the mitochondrial Mn-SOD (SOD2). This implies that Mn-SOD (SOD2) and PUMA but not Cu–Zn-SOD (SOD1) and CCS are involved in PRIMA-1-induced apoptosis in mutant p53 MCF-7 cells (Fig. 6).

4. Discussion

This report is the first to show that hypoxia increases PRIMA-1 toxicity against wt p53 MCF-7 cells. In contrast, another p53-reactivating agent, nutlin-3 that targets wt p53, was unable to activate wt p53 MCF-7 cells even under hypoxia (Fig. 4A and B). p53 reactivation by nutlin-3 involves interfering with the binding of MDM2 to the p53 N-terminus. However, MDM2 and its MDMX homolog show 80% similarity in their MDM2-p53-binding domains [23,24]. Since nutlin-3 does not disrupt p53-MDMX interaction, its ability to activate p53 appears compromised in tumors over-expressing MDMX like MCF-7 cells [23,24]. One possible mechanism to explain the hypoxia-mediated enhancement of PRIMA-1 toxicity in wt p53 MCF-7 breast carcinoma cells, is that hypoxia increases a mutant p53 conformation in these cells [13,15] (Fig. 4C). Another novel finding was that hypoxia increased the efficacy of PRIMA-1 against mutant p53 SKBR3 cells that over-express Her2/erbB2 (Fig. 3). This hypoxia-mediated enhancement of PRIMA-1 toxicity was counteracted by the anti-oxidant N-acetylcysteine, implying that increased oxidative stress under low oxygen further increases PRIMA-1 toxicity (Fig. 3A). SOD2 was increased by hypoxia and PRIMA-1 in wt p53 MCF-7 cells (Fig. 5A), implying that these joint treatments increased dismutation of superoxide into H_2O_2, in agreement with others demonstrating a role for H_2O_2 in hypoxic stress [7,6]. The importance of SOD2 in PRIMA-1 toxicity was further emphasized when Mn-SOD (SOD2) over-expressing Mn11-MCF-7 cells showed greater susceptibility to PRIMA-1 when compared to their neo4 control cells (Fig. 5B). This report also demonstrated that depletion of H_2O_2 by exogenous peroxidase enhanced PRIMA-1 toxicity and PUMA expression in MCF-7 cells transduced with mutant p53R175H [30]. No comparable potentiation was seen when PRIMA-1 was used together with exogenous SOD, implying that an imbalance in H_2O_2 may be more critical for the response to PRIMA-1. The fact that peroxidase synergizes with PRIMA-1 (Fig. 6) is compatible with results showing that distinct H_2O_2 concentrations are required to promote proliferation of tumor cells [6]. It was noteworthy that the synergism of PRIMA-1 plus exogenous peroxidase increased expression of the p53-dependent PUMA protein [20,21], and selectively induced the mitochondrial Mn-SOD (SOD2), possibly by increasing oxidative imbalance (Fig. 6). These results suggest that enzymatically diminishing exogenous H_2O_2 in the presence of PRIMA-1, may further increase mitochondrial H_2O_2 as a result of greater Mn-SOD expression (Fig. 6).

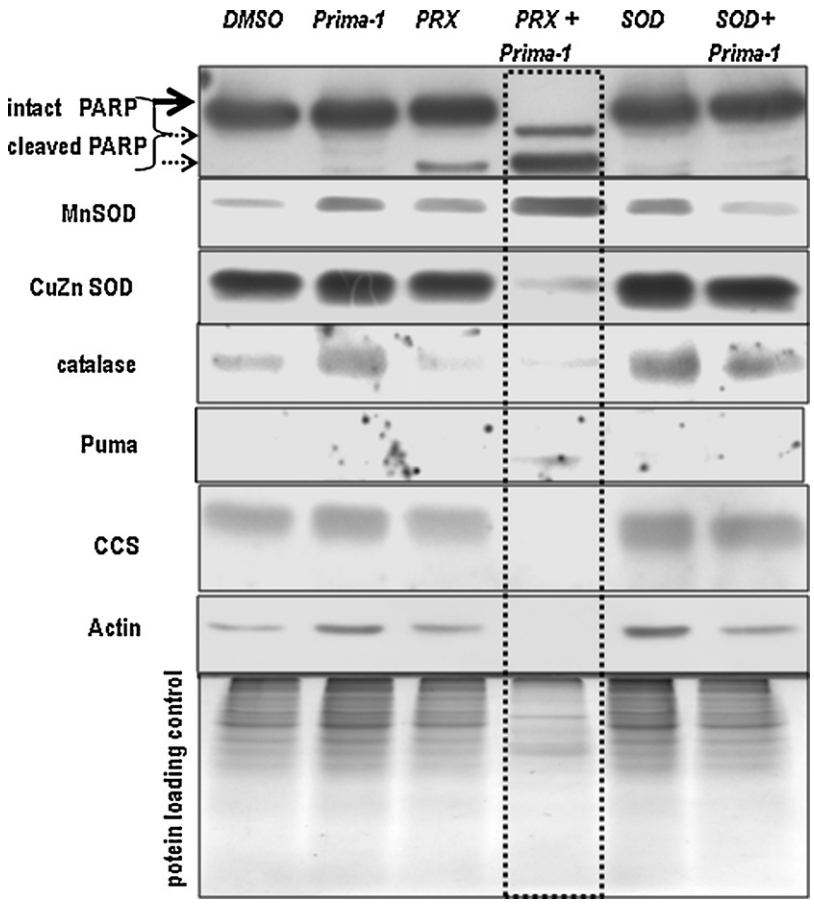

Fig. 6. PRIMA-1 synergizes with peroxidase to promote mut p53-MCF-7 apoptosis associated with induction of Mn-SOD and PUMA. Mutant p53-MCF-7 genetically matched to the wt p53 MCF-7 cells, described in Fig. 5A, were cultured overnight in complete medium, followed by a 24 h exposure to PRIMA-1, peroxidase, or SOD, whenever indicated. Subsequently, cells were harvested and subjected to electrophoretic separation and immune blotting, as indicated under Section 2. Note that apoptosis-associated PARP cleavage (small discontinuous arrows) and induction of Mn-SOD and PUMA are potentiated by joint treatment with PRIMA-1 and peroxidase (PRX).

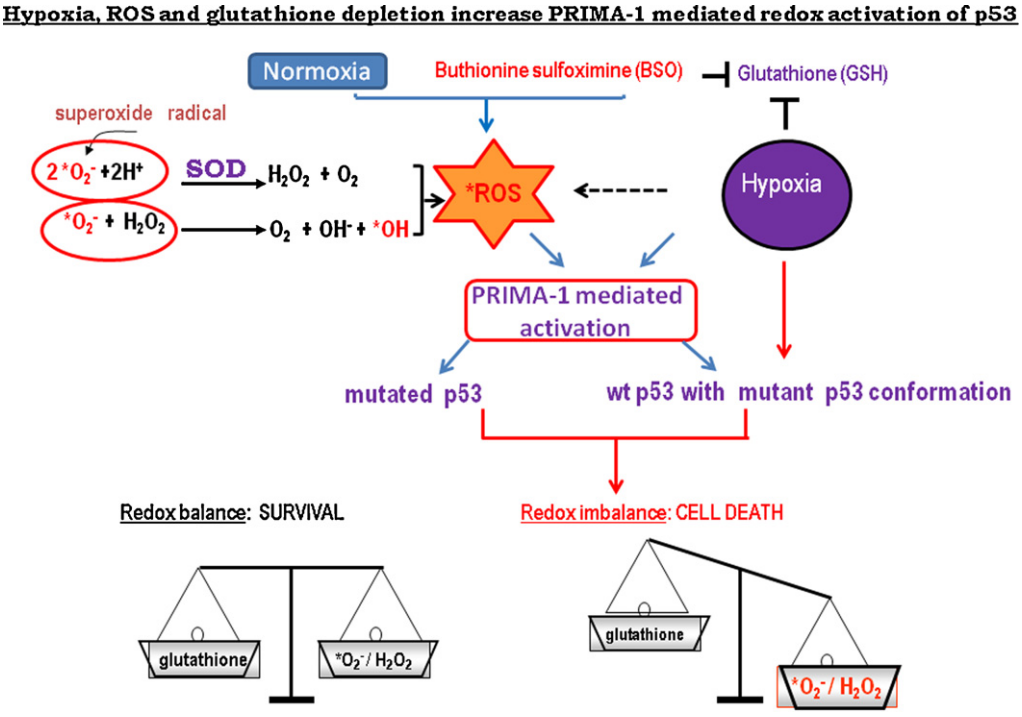

Fig. 7. Summary. Hypoxia, ROS and glutathione depletion increase PRIMA-1 mediated redox activation of p53.

5. Conclusions

Taken together, these results indicate for the first time that PRIMA-1 toxicity against wt p53 MCF-7 cells is increased under hypoxia. Another important finding is the enhancement of PRIMA-1 toxicity in normoxia when these cells over-express Mn-SOD. Glutathione depletion with BSO [35,36] did not affect the viability of neo[4] and Mn[11] MCF-7 cells, but potentiated PRIMA-1 toxicity against these cells under normoxia, irrespective of Mn-SOD over-expression. Hypoxia apparently increases unfolding of wt p53 to a more mutant-like conformation [13,15], which is likely to promote susceptibility to PRIMA-1, through a redox mechanism involving thiol groups in the p53 molecule [37] (see Fig. 7, summary). Survival under a hypoxic microenvironment for cancer cells like those used in this study, frequently correlates with tumor progression [1,2], inactivation of the p53 tumor suppressor gene [10,14,13,15] and diminished response to chemotherapy or radiation [1]. However, since hypoxia increases the efficacy of PRIMA-1 but not that of nutlin-3 against MCF-7 cells irrespective of p53 status, this report offers a novel therapeutic alternative to kill hypoxic wt p53 breast cancer tumors like MCF-7 cells, known to be resistant to other p53-reactivating agents like nutlin-3, because of MDMX over-expression [24,25].

Competing interests

The authors declare that they have no competing interests.

Authors' contribution

Mary Strasberg-Rieber contributed fundamentally to the acquisition and interpretation of data; and to the critical revision of the manuscript. Manuel Rieber conceived and designed acquisition of data, and was involved in drafting and critically revising the manuscript. All authors read and approved the final manuscript.

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References

[1] Onozuka H, Tsuchihara K, Esumi H. Hypoglycemic/hypoxic condition in vitro mimicking the tumor microenvironment markedly reduced the efficacy of anticancer drugs. Cancer Sci 2011;102(5):975–82.
[2] Louie E, Nik S, Chen JS, Schmidt M, Song B, Pacson C, et al. Identification of a stem-like cell population by exposing metastatic breast cancer cell lines to repetitive cycles of hypoxia and reoxygenation. Breast Cancer Res 2010;12(6):R94.
[3] Fandrey J, Frede S, Jelkmann W. Role of hydrogen peroxide in hypoxia-induced erythropoietin production. Biochem J 1994;303:507–10.
[4] Chandel NS, Maltepe E, Goldwasser E, Mathieu CE, Simon MC, Schumacker PT. Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. Proc Natl Acad Sci USA 1998;95:11715–20.
[5] .Sheldon RA, Christen S, Ferriero DM. Genetic and pharmacologic manipulation of oxidative stress after neonatal hypoxia–ischemia. Int J Dev Neurosci 2008;26(1):87–92.
[6] Schild L, Makarow P, Haroon F, Krautwald K, Keilhoff G. Distinct H_2O_2 concentration promotes proliferation of tumour cells after transient oxygen/glucose deprivation. Free Radic Res 2008;42(3):237–43.
[7] Vergara R, Parada F, Rubio S, Pérez F. Hypoxia induces H_2O_2 production and activates antioxidant defence system in grapevine buds through mediation of H_2O_2 and ethylene. J Exp Bot 2012;63(11):4123–31.
[8] Knappskog S, Lønning PE. P53 and its molecular basis to chemoresistance in breast cancer. Expert Opin Ther Targets 2012;16(Suppl. 1):S23–30.
[9] Naccarati A, Polakova V, Pardini B, Vodickova L, Hemminki K, Kumar R, et al. Mutations and polymorphisms in TP53 gene—an overview on the role in colorectal cancer. Mutagenesis 2012;27(2):211–8.
[10] Sermeus A, Michiels C. Reciprocal influence of the p53 and the hypoxic pathways. Cell Death Dis 2011;2:e164.
[11] Chavez-Perez VA, Strasberg-Rieber M, Rieber M. Metabolic utilization of exogenous pyruvate by mutant p53 (R175H) human melanoma cells promotes survival under glucose depletion. Cancer Biol Ther 2011;12(7):647–56.
[12] Madan E, Gogna R, Bhatt M, Pati U, Kuppusamy P, Mahdi AA. Regulation of glucose metabolism by p53: emerging new roles for the tumor suppressor. Oncotarget 2011;2(12):948–57.
[13] Gogna R, Madan E, Kuppusamy P, Pati U. Chaperoning of mutant p53 protein by wild-type p53 protein causes hypoxic tumor regression. J Biol Chem 2012;287(4):2907–14.
[14] Li J, Zhang X, Sejas DP, Bagby GC, Pang Q. Hypoxia-induced nucleophosmin protects cell death through inhibition of p53. J Biol Chem 2004;279:41275–79.
[15] Buizza L, Cenini G, Lanni C, Ferrari-Toninelli G, Prandelli C, Govoni S, et al. Conformational altered p53 as an early marker of oxidative stress in Alzheimer's disease. PLoS ONE 2012;7(1):e29789.
[16] Puca R, Nardinocchi L, Porru M, Simon AJ, Rechavi G, Leonetti C, et al. D'Orazi G. Restoring p53 active conformation by zinc increases the response of mutant p53 tumor cells to anticancer drugs. Cell Cycle 2011;10:1679–89.
[17] Bykov VJ, Issaeva N, Shilov A, Hultcrantz M, Pugacheva E, Chumakov P, et al. Restoration of the tumor suppressor function to mutant p53 by a low-molecular-weight compound. Nat Med 2002;8(3):282–8.
[18] Zache N, Lambert JM, Wiman KG, Bykov VJ. PRIMA-1MET inhibits growth of mouse tumors carrying mutant p53. Cell Oncol 2008;30:411–8.
[19] Zandi R, Selivanova G, Christensen CL, Gerds TA, Willumsen BM, Poulsen HS. PRIMA-1Met/APR-246 induces apoptosis and tumor growth delay in small cell lung cancer expressing mutant p53. Clin Cancer Res 2011;17(9):2830–41.
[20] Liang Y, Besch-Williford C, Hyder SM. PRIMA-1 inhibits growth of breast cancer cells by re-activating mutant p53 protein. Int J Oncol 2009;35:1015–23.
[21] Wang T, Lee K, Rehman A, Daoud SS. PRIMA-1 induces apoptosis by inhibiting JNK signaling but promoting the activation of Bax. Biochem Biophys Res Commun 2007;352(1):203–12.
[22] Lambert JM, Gorzov P, Veprintsev DB, Söderqvist M, Segerbäck D, Bergman J, et al. PRIMA-1 reactivates mutant p53 by covalent binding to the core domain. Cancer Cell 2009;15:376–88.
[23] Vassilev L, Graves B, Carvajal D, Podlaski F, Filipovic Z, Kong N, et al. In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. Science 2004;303:844–8.
[24] Hu B, Gilkes DM, Farooqi B, Sebti SM, Chen J. MDMX overexpression prevents p53 activation by the MDM2 inhibitor nutlin. J Biol Chem 2006;281:33,030–035.
[25] Wang H, Ma X, Ren S, Buolamwini JK, Yan C. A small-molecule inhibitor of MDMX activates p53 and induces apoptosis. Mol Cancer Ther 2011;10(1):69–79.
[26] Supiot S, Hill RP, Bristow RG. Nutlin-3 radiosensitizes hypoxic prostate cancer cells independent of p53. Mol Cancer Ther 2008;7(4):993–9.
[27] Bossi G, Lapi E, Strano S, Rinaldo C, Blandino G, Sacchi A. Mutant p53 gain of function: reduction of tumor malignancy of human cancer cell lines through abrogation of mutant p53 expression. Oncogene 2006;25:304–9.
[28] Viola-Rhenals M, Rieber MS, Rieber M. Suppression of survival in human SKBR3 breast carcinoma in response to metal–chelator complexes is preferential for copper-dithiocarbamate. Biochem Pharmacol 2006;71(6):722–34.
[29] Camerini A, Donati S, Viacava P, Siclari O, Puccetti C, Tartarelli G, et al. .Evaluation of HER2 and p53 expression in predicting response to docetaxel-based first-line chemotherapy in advanced breast cancer. J Exp Clin Cancer Res 2011;30:38.
[30] Rieber M, Strasberg Rieber M. DN-R175H p53 mutation is more effective than p53 interference in inducing epithelial disorganization and activation of proliferation signals in human carcinoma cells: role of E-cadherin. Int J Cancer 2009;125:1604–12.
[31] Zhang HJ, Zhao W, Venkataraman S, Robbins ME, Buettner GR, Kregel KC, et al. Activation of matrix metalloproteinase-2 by overexpression of manganese superoxide dismutase in human breast cancer MCF-7 cells involves reactive oxygen species. J Biol Chem 2002;2(77):20,919–926.
[32] Kaewpila S, Venkataraman S, Buettner GR, Oberley LW. Manganese superoxide dismutase modulates hypoxia-inducible factor-1 alpha induction via superoxide. Cancer Res 2008;68(8):2781–8.
[33] Rhenals MV, Strasberg-Rieber M, Rieber M. Nitric oxide donors or nitrite counteract copper-[dithiocarbamate](2)-mediated tumor cell death and inducible nitric oxide synthase down-regulation: possible role of a nitrosylcopper [dithiocarbamate](2) complex. J Med Chem 2010;53:1627–35.
[34] Rieber M, Rieber MS. Mcl-1 cleavage and sustained phosphorylation of c-Jun-N-terminal kinase mediate melanoma apoptosis induced by 2-acetyl furanonaphthoquinone: roles of Bcl-2 and p53. Cancer Biol Ther 2008;7(8):1206–11.
[35] Ogunrinu TA, Sontheimer H. Hypoxia increases the dependence of glioma cells on glutathione. J Biol Chem 2010;285(48):37,716–724.
[36] Magwere T, Burchill SA. Heterogeneous role of the glutathione antioxidant system in modulating the response of ESFT to fenretinide in normoxia and hypoxia. PLoS ONE 2011;6(12):e28558.
[37] Bykov VJ, Lambert JM, Hainaut P, Wiman KG. Mutant p53 rescue and modulation of p53 redox state. Cell Cycle 2009;8:2509–17.